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The MultiBac System: A Perspective

Imre Berger[1,2*], Christine Tölzer[1], Kapil Gupta[1]

[1] Bristol Synthetic Biology Centre BrisSynBio, Biomedical Sciences, School of Biochemistry, University of Bristol, 1 Tankard's Close, Bristol BSH 1TD, United Kingdom

[2] Max Planck Bristol Centre for Minimal Biology, Cantock's Close, Bristol BS8 1TS, United Kingdom

[*] Correspondence to: imre.berger@bristol.ac.uk

Abstract

Baculovirus expression is a time-tested technique to produce proteins in insect cells, in high quality and quantity for a range of applications. MultiBac is a baculovirus expression system we developed originally for producing multiprotein complexes comprising many subunits, for structural and mechanistic studies. First introduced in 2004, MultiBac is now in use in many laboratories world-wide, accelerating research programmes in academia and industry. We have continuously optimized our MultiBac system, providing customized reagents and standard operating protocols to facilitate its use also by non-specialists. More recently, we have generated MultiBac genomes tailored for specific purposes, for example to produce humanized glycoproteins, high-value pharmaceutical targets including kinases, viral polymerases, and virus-like particles (VLPs) as promising vaccine candidates. By altering the host tropism of the baculovirion, we created MultiBacMam, a heterologous DNA delivery toolkit to target mammalian cells, tissues and organisms. Introducing CRISPR/Cas modalities, we set the stage for large-scale genomic engineering applications utilizing this high capacity DNA delivery nanodevice. Exploiting synthetic biology approaches and bottom-up design, we engage in optimizing the properties of our baculoviral genome, also to improve manufacturing at scale. Here we provide a perspective of our MultiBac system and its developments, past, present and future.

Summary Points

- MultiBac system unlocks structure and mechanism of multiprotein complexes.
- Non-integrative DNA delivery system with unprecedented cargo capacity.
- Host cell tropism can be engineered to target mammalian cells, tissues, and organisms.
- Potential replacement technology for current viral vectors in gene therapy.

Main Text

MultiBac Origins: The Nuts and Bolts

Our interest in the baculovirus expression system dates from our work at the Swiss Federal Institute of Technology, ETH Zürich, in the laboratory of Tim Richmond, an expert in chromatin biology. It was the late 1990s and Tim had just determined the high-resolution structure of the nucleosome core particle, a seminal contribution to structural biology and a definite milestone in our understanding of chromatin architecture [1]. Nucleosomes comprise eight histone proteins organised in the so-called histone octamer, around which wraps a DNA superhelix. The Richmond team had produced all components recombinantly. Nucleosomes are the central building units of chromatin, and a host of protein factors engage with this key component of the hereditary material. Many of these factors are multiprotein complexes, often comprising 10 or more subunits. The low abundance of these vital protein specimens, exacerbated by compositional heterogeneity, typically impeded their extraction from native source material in the quality and quantity required for detailed mechanistic studies. In the case of the nucleosome, recombinant production of the components had fostered the breakthrough enabling near atomic structure determination. Consequently, we endeavoured to produce our protein complexes of interest similarly, using recombinant overproduction techniques.

This, however, was easier said than done. Back then, the recombinant toolbox to produce complexes, was fairly rudimentary, to say the least. Existing technology was mostly confined to produce small, binary complexes in *E. coli*, the common prokaryotic host. Of note, the components of the nucleosome had been produced as inclusion bodies, and then reconstituted by refolding, an approach wholesale unfeasible for the much larger eukaryotic subunits, many of them with multidomain architecture, in our complexes of interest totalling up to and beyond a megadalton in combined molecular weight. We spent a rather depressing aggregate of post-doc years in trying most of not all recombinant expression systems then available, including

Pichia pastoris, a yeast which was the hot 'kid-in-the-block' for eukaryotic protein production in those days. Unfortunately, all our efforts to produce the complexes we wished to investigate failed, soundly and comprehensively.

Things took a turn to the better, however. Tim had met Jane Endicott, an expert kinase structural biologist then at Oxford University. Jane used baculovirus expression prolifically to produce the kinases they crystallized, and she kindly offered to help out by training one of us in her laboratory. Very soon, the lead author of the present article spent two wonderful weeks in Oxford with the Endicott team, learning the basics of the trade. Incidentally, a leading expert in baculovirus technology worked at that time in the nearby virology institute, Polly Roy. Jane organized a meeting, and Polly in her very compelling way instructed us in what, in her view, was the solution to all our problems: "You must place all recombinant genes on one single baculovirus and then you have your complex." Back in Zürich, thoroughly inspired and motivated, we rolled up our sleeves to develop the reagents and protocols that culminated in the MultiBac system (Figure 1) [2-11].

Our initial efforts focused on devising a way to simplify and standardize combination of the many genes encoding for the complex subunits into multigene expression constructs and then to introduce these in a straight forward manner into the baculoviral genome. We started from a baculoviral genome available from Invitrogen, which exists in from of a bacterial artificial chromosome (BAC). We provided a set of small transfer plasmids comprising multiplication modules and site-specific recombination elements to assemble multigene expression cassettes [2, 4, 5]. To facilitate DNA assembly, we utilized sequence and ligation independent protocols in combination with plasmid fusion in a process we termed tandem recombineering (TR) [12], which we subsequently implemented in high throughput in a robotics set-up [8, 13, 14]. In addition to a Tn7 attachment site for transposon mediated heterologous DNA insertion, we also engineered a DNA sequence encoding a loxP site into the viral backbone, to allow integration

by Cre recombinase-mediated plasmid fusion of additional functionalities (e.g. phosphatases, kinases, glycosylases, deglycosylases, chaperones etc.) to modify, activate or inactivate a particular protein specimen produced from the Tn7 site on the same baculoviral genome [6, 15]. This additional site proved to be highly useful for generating customized MultiBac baculoviral genomes for specific applications (Figure 1) [10, 16-22]. All along, our ambition was to streamline the handling of the entire system, including composite baculovirus generation, tissue culture, virus amplification and protein complex production, to render the technique accessible to a wide range of users including non-specialists who would only occasionally use this tool. The insertion of a yellow fluorescent protein as a marker proved particularly useful – by monitoring the fluorescent signal, or simply by looking at the colour of the culture turning greenish, we could determine when cultures needed to be harvested to obtain best results [4].

Immediately, the MultiBac system was well received in the community and the interest appears to continue unabated since. Tantalizing structures have been determined, revealing exciting mechanisms (selection shown in Figure 1), incidentally including structures of chromatin interacting protein complexes bound to nucleosomes of the kind for which the system was originally envisaged [23-26]. It is probably fair to say, and foremostly gratifying, that MultiBac has become a system of choice for producing eukaryotic multiprotein complexes for structural and functional studies at high resolution by X-ray crystallography and electron cryo-microscopy.

Together with Dan Fitzgerald, a fellow post-doc in Zürich, we installed a small facility in the basement of ETH's molecular biology building, to operate MultiBac as a platform physically separate from all yeast and *E.coli* expressions in the department, with the aim to forestall contamination which had plagued our work in the initial stages when we had been less rigorous in our handling. This first 'MultiBac facility' was an improvised enterprise – we had

decided not to use incubators, but to temperature the entire room to 27°C, using a makeshift set-up of modified shakers, fans and thermostats bought from the supermarket which we combined with a feed-back loop to keep the temperature within the narrow range tolerated by the insect cells we cultured. It soon became our favourite locality in the building, remote and cosy to work in. However, in spite of great efforts to impose and maintain good and sterile laboratory practice, we noticed bouts of contamination in the facility, recurring exactly every third week-end in a month. It caused some headache to track down the source of this calamity. It turned out to be yeast, and moreover to coincide precisely with the departmental Happy Hour scheduled exactly at the same intervals, where quite naturally significant quantities of beer were consumed. We reacted by ruling that use of the MultiBac facility and participating in Happy Hour were mutually exclusive, and our contamination issue was swiftly resolved.

Broadening the scope: MultiBac moves to EMBL

Soon the MultiBac platform migrated together with the Berger team to the European Molecular Biology Laboratory, EMBL Grenoble. Together with facility manager Fred Garzoni, we set up a much larger facility serving the sizeable structural biology community in Grenoble and beyond [27]. We participated in transnational access programmes bringing many scientists from diverse backgrounds to our laboratory to learn the technique, based on their own projects. This was an intensive and horizon broadening experience for us. We were exposed to a wide range of projects and realized that the baculovirus system, and specifically MultiBac, had a significantly broader scope beyond structural biology [3]. Applications which stood out were the use of our system to produce influenza polymerase, a long elusive protein complex controlling replication and transcription of this pathogen [28]. In a nature-inspired approach, we utilized a single ORF encoding all subunits of the polymerase within a polyprotein, cleaved apart co-translationally by a highly specific protease likewise encoded in the ORF, to unlock this high-value drug target to high-resolution structure analysis [29, 30]. A further highlight

was MultiBac-produced recombinant adeno-associated virus (AAV) for gene therapy of obesity in a rodent model [3, 31]. This triggered our keen interest, prompting us to invest in developing the system towards new and exciting applications [9, 17].

Synthetic Biology: The MultiBac platform in Bristol

Our MultiBac platform in its current embodiment is operating at the University of Bristol, UK, underpinning research and development in the Bristol Synthetic Biology Centre, BrisSynBio, and the new Max Planck Bristol Centre for Minimal Biology inaugurated in March 2019. Game-changing developments in custom DNA synthesis and the concomitant remarkable reduction in costs set the stage for revisiting all aspects of our technology for improvement. We are close to the point where cloning heterologous gene expression cassettes and their conjoining into multigene circuitry can be confidently outsourced to commercial suppliers, making redundant much of the DNA assembly routines in research laboratories. Moreover, the total synthesis of a baculoviral genome in a bottom-up approach is in principle now possible. The baculovirus we use in cell culture exists in a narrowly defined, confined habitat rendering a significant portion of the genomic content probably superfluous. For instance, genes regulating virus *per os* uptake, and modalities providing long-term protection against exposure can conceivably be disposed of. By data mining and comparative genomics, we provided a blueprint for a minimal, condensed baculoviral genome which we are assembling in a hybrid approach, iteratively grafting synthetic DNA segments we rewired on the MultiBac genome replacing wild-type sequence [9, 32]. Our objective here is to alleviate detrimental features of the baculovirus, notably its genomic instability during serial passage for amplification which poses an impediment for manufacturing at pharmaceutically scale. At the same time, eliminating instability hotspots and superfluous genetic information from the baculoviral genome will further increase its already remarkable heterologous DNA cargo capacity (in our hands 100 kb). This unprecedented DNA cargo capacity is particularly

interesting when combined with reconfiguring the host tropism of baculovirus which in nature is narrowly confined for specific insect cells. It was shown that by pseudotyping for example with vesicular stomatitis virus glycoprotein, baculovirus can be reconfigured into a DNA delivery tool for mammalian cells and tissues [33, 34]. We seized this opportunity and created MultiBacMam, a MultiBac baculovirus-based transient or integrative delivery system with unprecedented cargo capacity for heterologous DNA circuitry (Figure 1) [17, 19].

Gene therapy today is dominated by lentivirus, adenovirus and adeno-associated virus, each with their own merits and shortcomings. In our view, baculovirus has the potential to become a highly attractive replacement technology for current viral vectors, based on its far superior DNA cargo capacity, the ease of engineering, and much simpler manufacturing, particularly once the genomic instability issues are resolved. Moreover, baculovirus is non-integrative, does not scar the host genome and, importantly, does not replicate in a mammalian host. We anticipate rapid progress and exciting breakthroughs in the near future, in baculovirus development towards a high capacity delivery system for therapeutic genome engineering, potentiated by powerful DNA insertion methodologies including CRISPR/Cas. The race is on - and remains exciting.

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Author Contributions

I.B., C.T. and K.G. prepared the manuscript together.

Competing Interest Statement

IB declares competing interest. IB is inventor on patents and patent applications relating to the MultiBac technology and is co-founder and shareholder of biotech companies commercializing MultiBac and its applications.

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Figure and Legend

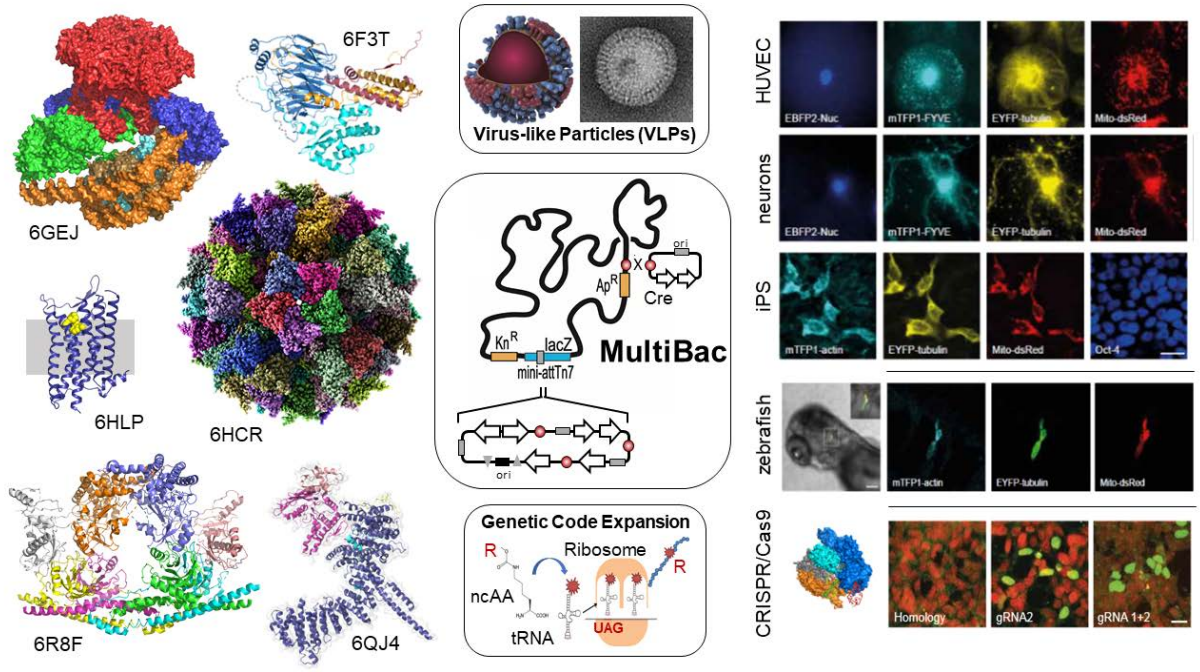

Figure 1. MultiBac Applications. The MultiBac system is depicted schematically in the centre. It consists of an engineered baculoviral genome which exists as a bacterial artificial chromosome (BAC) in specialized bacterial cells. Foreign DNA can be introduced into this BAC using a set of helper plasmids comprising the multigene circuitry of choice, by a variety of means including Tn7 mediated transposition and/or site directed imsertion by Cre recombinase [2-5, 8, 10, 11]. Originally, we designed the MultiBac system for expressing heterologous multiprotein complexes in insect cells. A selection of recent high-impact structures of samples produced using MultiBac is shown on the left, marked by their Protein Data Bank identifier (PDB-ID). 6GEJ, SWR1-nucleosome complex [25]; 6F3T, human TFIID subcomplex TAF5-TAF6-TAF9 [35]; 6HLP, human neurokinin 1 receptor GPCR [36]; 6R8F, human BRISC-SHMT2 complex [37]; 6QJ4, Ycs4-Brn1/Smc4hd–Brn1C complex [38]; 6HCR, ADDomer virus-like particle (VLP). MultiBac is successfully used for a range of applications beyond producing protein complexes for structural and mechanistic studies. For example, customized MultiBac baculoviral genomes were prepared to express virus-like

particles (VLPs), which are promising vaccine candidates as they resemble live viruses but do not contain genetic material and are thus safe [22, 39]. Here, an influenza VLP is shown in a schematic drawing and in an electron micrograph [22] (centre, top). A different example is MultiBacTAG, a customized MultiBac baculovirus capable of genetic code expansion [18] (centre, bottom). MultiBacTag exploits an orthogonal tRNA/tRNA synthetase pair to insert artificial amino acids (ncAA) into polypeptide chains at defined sites by means of AMBER codon (UAG) suppression [18, 40]. By altering the tropism of the baculovirion, MultiBac can be turned into a powerful high-capacity DNA delivery device into mammalian cells, tissues and even organisms (right), to faithfully deliver multicomponent DNA circuitry transiently or stably by genomic insertion using CRISPR/Cas technology [9, 17, 19, 41]. For a more detailed conspectus of MultiBac applications see recent review articles by our group [9, 10, 17].